17 DEC 1998

FORM PTO-1390 (REV. 1-98) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OF

# TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

| ATTORNEY'S DOCKET NUMBER | |
|---|---|
| 20-4491P | |
| U.S. APPLICATION NO. (If known, see 37 CFR 1.5) | |
| NEW | |

| INTERNATIONAL APPLICATION NO. | INTERNATIONAL FILING DATE | PRIORITY DATE CLAIMED |
|---|---|---|
| PCT/JP97/01893 | June 4, 1997 | June 7, 1996 |

TITLE OF INVENTION
TUMOR ANTIGEN PROTEINS, GENES THEREFOR, AND TUMOR ANTIGEN PEPTIDES

APPLICANT(S) FOR DO/EO/US
ITOH, Kyogo; SHICHIJO, Shigeki; IMAI, Yasuhisa

**Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:**

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39 (1).
4. ☐ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date
5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. ☐ is transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☒ has been transmitted by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☒ A translation of the International Application into English (35 U.S.C. 371(c)(3)).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(2)).
   a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☐ have been transmitted by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☒ have not been made and will not be made.
8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

**Items 11. to 16. below concern document(s) or information included:**

11. ☒ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. ☒ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ☒ A FIRST preliminary amendment.
    ☐ A SECOND or SUBSEQUENT preliminary amendment.
14. ☐ A substitute specification.
15. ☐ A change of power of attorney and/or address letter.
16. ☒ Other items or information:
    1). Receipt in the case of of an original deposit of microorganisms, and translation (3 total)
    2) 1 sheet of Formal Drawings.
    3). PCT/ISA/210 International Search Report.

| U.S. APPLICATION NO (if known, see 37 CFR 1.5) | INTERNATIONAL APPLICATION NO | ATTORNEY'S DOCKET NUMBER |
|---|---|---|
| NEW | PCT/JP97/01893 | 20-4491 |

17. ☒ The following fees are submitted:

| | CALCULATIONS | PTO USE ONLY |
|---|---|---|
| **BASIC NATIONAL FEE (37 CFR 1.492(a)(1)-(5):** | | |
| Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO ..... $970.00 | | |
| International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO ..... $930.00 | | |
| International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO ..... $760.00 | | |
| International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) ..... $670.00 | | |
| International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) ..... $96.00 | | |
| **ENTER APPROPRIATE BASIC FEE AMOUNT =** | $ 930.00 | |
| Surcharge of $130.00 for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(e)). | $ 0.00 | |

| CLAIMS | NUMBER FILED | NUMBER EXTRA | RATE | | |
|---|---|---|---|---|---|
| Total Claims | 28 - 20 = | 8 | X $18.00 | $ 144.00 | |
| Independent Claims | 2 - 3 = | 0 | X $78.00 | $ 0.00 | |
| MULTIPLE DEPENDENT CLAIM(S) (if applicable) | | YES | + $260.00 | $ 260.00 | |
| ☐ | | | **TOTAL OF ABOVE CALCULATIONS =** | $ 1,334.00 | |
| Reduction of ½ for filing by small entity, if applicable. Verified Small Entity statement must also be filed (Note 37 CFR 1.9, 1.27, 1.28). | | | | $ 0.00 | |
| ☐ | | | **SUBTOTAL =** | $ 1,334.00 | |
| Processing fee of $130.00 for furnishing the English translation later than ☐20 ☐30 months from the earliest claimed priority date (37 CFR 1.492(f)). | | | + | $ 0.00 | |
| | | | **TOTAL NATIONAL FEE =** | $ 1,334.00 | |
| Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). $40.00 per property | | | + | $ 40.00 | |
| | | | **TOTAL FEES ENCLOSED =** | $ 1,374.00 | |
| | | | | **Amount to be: refunded** | $ |
| | | | | **charged** | $ |

a. ☒ A check in the amount of $ 1,374.00 to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account. No. _____ in the amount of $_____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 02-2448.

**NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.**

#32,881

Send all correspondence to:
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SIGNATURE

MURPHY, GERALD M., JR.
NAME

28,977
REGISTRATION NUMBER

/mm December 7, 1998

20-4491P

IN THE U.S. PATENT AND TRADEMARK OFFICE

APPLICANT: Kyogo ITOH et al.

INT'L. APPLN. NO.: PCT/JP97/01893

SERIAL NO.: NEW GROUP:

FILED: December 7, 1998 EXAMINER:

FOR: TUMOR ANTIGEN PROTEINS, GENES THEREFOR, AND TUMOR ANTIGEN PEPTIDES

PRELIMINARY AMENDMENT

Assistant Commissioner of Patents
and Trademarks
BOX PATENT APPLICATION
Washington, D.C. 20231

December 7, 1998

Sir:

The following Preliminary Amendments and Remarks are respectfully submitted in connection with the above-identified application.

IN THE SPECIFICATION:

Before line 1, insert --This application is the national phase under 35 U.S.C. §371 of prior PCT International Application No. PCT/JP97/01893 which has an International filing date of June 4, 1997 which designated the United States of America.--

**IN THE CLAIMS:**

Claim 9, lines 2 and 3, please delete ",the tumor antigen peptide or derivative thereof defined in claim 7 or 8"

Claim 10, line 2, please delete "or the tumor antigen peptide of claim 7 or 8"

Please Add the following new claims.

--12. A medicine comprising, as an active ingredient, the tumor antigen peptide or derivate thereof as defined in claim 7.

13. A medicine comprising, as an active ingredient, the tumor antigen peptide or derivate thereof as defined in claim 8.

14. An antibody which specifically binds to the tumor antigen peptide of claim 7.

15. An antibody which specifically binds to the tumor antigen peptide of claim 8.--

## R E M A R K S

The specification has been amended to provide a cross-reference to the previously filed International Application.

Claims 9-10 have been amended and claims 12-15 added to remove improper multiple dependices in the original claims.

If necessary, the Commissioner is hereby authorized in this, concurrent, and future replies, to charge payment or credit any overpayment to Deposit Account No. 02-2448 for any additional fees required under 37 C.F.R. §1.16 or under 37 C.F.R. §1.17; particularly, extension of time fees.

Respectfully submitted,

BIRCH, STEWART, KOLASCH & BIRCH, LLP

By _____ #32,801

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(Rev. 11/16/98)

# DESCRIPTION

## Tumor Antigen Proteins, Genes Therefor, and Tumor Antigen Peptides

## TECHNICAL FIELD

The present invention relates to medicines for activating antitumor immunity and for treating autoimmune diseases as well as to diagnosis of tumors or autoimmune diseases. In particular, the present invention relates to novel tumor antigen proteins, novel genes therefor, novel tumor antigen peptides, and the like.

## PRIOR ART

It is known that the immune system, particularly T cells, plays an important role *in vivo* in tumor rejection. Indeed, infiltration of lymphocytes having cytotoxic effects on tumor cells has been observed in human tumor foci (*Arch. Surg.*, **126**:200-205, 1990), and cytotoxic T lymphocytes (CTLs) recognizing autologous tumor cells have been isolated from melanomas without great difficulties (e.g., *Immunol. Today*, **8**:385, 1987; *J. Immunol.*, **138**:989, 1987; and *Int. J. Cancer*, **52**:52-59, 1992). In addition, the results of clinical treatment of melanomas by T cell introduction also suggest the importance of T cells in tumor rejection (*J. Natl. Cancer. Inst.*, **86**:1159, 1994).

Although it has long been unknown about target molecules for CTLs attacking autologous tumor cells, the recent advance in immunology and molecular biology has gradually begun elucidating such target molecules. Specifically, it has been found that using T cell receptors (TCRs), CTL recognizes a complex consisting of tumor antigen peptide and major histocompatibility complex (MHC) class I

antigen, and thereby attacks autologous tumor cells.

Tumor antigen peptides are generated from tumor antigen proteins. Thus, the proteins are intracellularly synthesized and then degraded in cytoplasm into the peptides by proteasome. On the other hand, MHC class I antigens formed at endoplasmic reticulum bind to the above tumor antigen peptides, and are transported via cis Golgi to trans Golgi, *i.e.*, the mature side, and expressed on the cell surface (*Rinsho-Menneki*, **27**(9):1034-1042, 1995).

As such a tumor antigen protein, T. Boon *et al.* identified a protein named MAGE from human melanoma cells for the first time in 1991 (*Science*, **254**:1643-1647, 1991), and thereafter several additional tumor antigen proteins have been identified from melanoma cells.

As described in the review by T. Boon *et al.* (*J. Exp. Med.*, **183**, 725-729, 1996), tumor antigen proteins hitherto identified can be divided into the following four categories.

Tumor antigen proteins belonging to the first category are those proteins which are expressed only in testis among normal tissues, while they are expressed in melanoma, head and neck cancer, non-small cell lung cancer, bladder cancer and others, among tumor tissues. Among tumor antigen proteins in this category are the above-described MAGE and analogous proteins constituting a family of more than 12 members (*J. Exp. Med.*, **178**:489-495, 1993), as well as BAGE (*Immunity*, **2**:167-175, 1995) and GAGE (*J. Exp. Med.*, **182**:689-698, 1995), all of which have been identified from melanoma cells.

Although some of such tumor antigen proteins in this category

are highly expressed in melanoma, their expression is observed in only 10 to 30% of patients having a particular tumor other than melanoma, and therefore, they can not be applied widely to treatments or diagnoses of various tumors.

Tumor antigen proteins belonging to the second category are those proteins which are expressed only in melanocytes and retina among normal tissues, while the expression is observed only in melanomas among tumor tissues. Since these tissue-specific proteins are highly expressed in melanomas, they function as tumor antigen proteins specific for melanomas. Among tumor antigen proteins in this category are tyrosinase (*J. Exp. Med.*, **178**:489-495, 1993), MART-1 (*Proc. Natl. Acad. Sci. USA*, **91**:3515, 1994), gp100 (*J. Exp. Med.*, **179**:1005-1009, 1994), and gp75 (*J. Exp. Med.*, **181**:799-804, 1995), genes for which have all been cloned from melanoma cells. Additionally and separately identified Melan-A (*J. Exp. Med.*, **180**:35, 1994) has proved to be the same molecule as MART-1.

However, the tumor antigen proteins in this category can not be used widely for treatments or diagnoses of various tumors, since they are not expressed in tumors other than melanoma.

Tumor antigen proteins belonging to the third category are those proteins which yield, through tumor-specific mutations, tumor antigen peptides newly recognized by CTL. Among tumor antigen proteins in this category are mutated CDK4 (*Science*, **269**:1281-1284, 1995), β-catenin (*J. Exp. Med.*, **183**:1185-1192, 1996), and MUM-1 (*Proc. Natl. Acad. Sci. USA*, **92**:7976-7980, 1995). In CDK4 and β-

catenin, a single amino acid mutation increases the binding affinity of the peptides to MHC class I antigen, and allows them to be recognized by T cells. In MUM-1, its intron normally untranslated is translated due to mutation, and the peptide thus generated is recognized by T cells. However, since such mutations occur at low frequency, they can not be applied widely to treatments or diagnoses of various tumors.

As a tumor antigen protein belonging to the fourth category, P15 has been identified from melanoma cells, which is a protein widely expressed in normal tissues and which is also recognized by CTL (*J. Immunol.* **154**:5944-5955, 1995).

Tumor antigen proteins or peptides hitherto known have been identified as follows.

In such identification, a set of tumor cells and CTLs attacking the tumor cells (usually established from lymphocytes of the same patient from whom the tumor cells are obtained) are firstly provided. Then, the cells from this set are used to directly identify tumor antigen peptides, or used to determine the gene encoding the tumor antigen protein from which corresponding tumor antigen peptides are identified.

Specifically, in the case where tumor antigen peptides are directly identified, tumor antigen peptides bound to MHC class I antigens in tumor cells are extracted under acidic conditions, and separated into various peptides using high-performance liquid chromatography. Cells expressing MHC class I antigen, but not expressing tumor antigen protein (for example, B cells from the same patient), are then pulsed with such various peptides, and examined for

their reactivity with CTL to identify tumor antigen peptides. Then, the sequences of the peptides thus identified are further determined by, for example, mass spectrometry. In this way, tumor antigen peptides derived from Pmel 17 which is the same molecule as gp100 have been identified from melanoma cells (*Science*, **264**:716-719, 1994).

In order to firstly determine the gene encoding tumor antigen protein and then to identify therefrom corresponding tumor antigen peptides, the gene encoding tumor antigen protein may be cloned using molecular biological techniques. cDNAs are prepared from tumor cells, and cotransfected with MHC class I antigen gene into cells not expressing tumor antigen proteins (for example, COS cells), in order to express them transiently. The products thus expressed are then repeatedly screened on the basis of their reactivity with CTL, until the gene encoding tumor antigen protein may finally be isolated. In this way, the genes for the above-mentioned MAGE, tyrosinase, MART-1, gp100, and gp75 have been cloned.

In order to deduce and identify the presented tumor antigen peptides actually bound to MHC class I antigens on the basis of the information about such tumor antigen gene, the methods as described below are used. Firstly, fragments of the gene encoding tumor antigen protein, having various sizes, are prepared using, for example, PCR, exonucleases, or restriction enzymes, and cotransfected with MHC class I antigen gene into cells not expressing tumor antigen proteins (e.g., COS cells), in order to express them transiently. The region(s) which include tumor antigen peptides are then identified on the basis of their

reactivity with CTL. Subsequently, peptides are synthesized. Cells expressing MHC class I antigen but not expressing tumor antigen proteins are then pulsed with the synthesized peptides, and examined for their reactions with CTL to identify the tumor antigen peptides (*J. Exp. Med.*, **176**:1453, 1992; *J. Exp. Med.*, **179**:24, 759, 1994). The sequence regularities (motifs) for peptides, which are bound and presented by certain types of MHC such as HLA-A1, -A0201, -A0205, -A11, -A31, -A6801, -B7, -B8, -B2705, -B37, -Cw0401, and -Cw0602 have been known (*Immunogenetics*, **41**:178-228, 1995), and therefore, candidates for tumor antigen peptides may also be designed by making reference to such motifs, and such candidate peptides may be practically synthesized and examined in the same way as described above (*Eur. J. Immunol.*, **24**:759, 1994; *J. Exp. Med.*, **180**:347, 1994).

Furthermore, it is another possibility that tumor antigen proteins expressed at high level in tumors are expressed also in normal tissues, and cause autoimmune diseases by inducing excessive immune response against such tumor antigen proteins. For example, it was reported that when a combination of a chemotherapeutic agent and IL-2 was used for treating melanomas, appearance of leukoderma was observed (*J. Clin. Oncol.*, **10**:1338-1343, 1992). This is probably because CTLs or antibodies against the complexes consisting of fragments of the tumor antigen protein expressed in melanomas (referred to as peptide fragments) and MHC class I antigens were inductively produced, and they affected normal skin tissues to develop leukoderma, an autoimmune disease-like symptom.

## SUBJECT THAT THE INVENTION IS TO SOLVE

As described above, some of the known tumor antigen proteins are expressed only in limited tumors, and others are expressed only in a small number of patients having a particular tumor even if they are expressed in various kinds of tumor, and threfore, they can not be used widely for treatments or diagnoses of various tumors.

Thus, the present invention aims to provide tumor antigen proteins or fragments thereof (hereinafter referred to as "peptide fragments" or as "tumor antigen peptides") which, unlike the known tumor antigen proteins or their peptide fragments, can be used for treatments or diagnoses of a wide variety of tumors including squamous cell carcinoma, or which can be applied to major part of patients having a particular tumor even if they can be used only for limited tumors, or which can be applied to various tumors as a therapeutic or diagnostic assistant in the treatment or diagnosis for such tumors.

Squamous cell carcinoma is one of the most common cancers in human. In particular, squamous cell carcinomas in esophageal cancer and lung cancer are known to be relatively resistant to current chemotherapy and radiotherapy. Also in this regard, it is desired to develop specific immunotherapies such as those which use tumor antigen proteins or corresponding tumor antigen peptides.

Furthermore, when one develops autoimmune disease due to excessively induced specific immunity raised by tumor antigen protein, it would be desirous treatments to specifically block such immune response using, for example, antisense DNA/RNA for the gene encoding tumor

antigen proteins or antagonists for the tumor antigen peptides.

MEANS FOR SOLVING THE SUBJECT

With the aim of obtaining tumor antigen protein or corresponding tumor antigen peptides which can be applied widely to treatments or diagnoses of various tumors including, in particular, squamous cell carcinoma, the present inventors tried to identify tumor antigen proteins from tumors other than melanoma.

Specifically, the present inventors established a squamous cell carcinoma cell line KE-4 derived from esophageal cancer (hereinafter referred to as esophageal cancer cell line KE-4 or simply as KE-4), and also established CTL (hereinafter referred to as KE-4CTL) which recognizes tumor antigen peptides restricted to HLA-A2601 which is a MHC class I antigen expressed in said KE-4 (*Cancer Res.*, **55**:4248-4253, 1995).

Fibroblast cell line VA-13 was then cotransfected with a recombinant plasmid of cDNA library prepared from KE-4 and a recombinant plasmid containing HLA-A2601 cDNA. The resulting transfectants were treated with KE-4CTL, and screened by measuring the amount of produced IFN-γ to determine whether KE-4-CTL was activated. As a result, the inventors succeeded in cloning a novel gene encoding tumor antigen protein of the present invention for the first time from tumor cells other than melanoma.

Thus, the gist of the present invention relates to :

(1) DNA encoding a protein having the amino acid sequence shown in SEQ ID NO: 1 or a variant protein thereof in which one or more amino

acid residues are substituted, deleted or added, said protein and variant protein thereof being capable of yielding, through its intracellular decomposition, peptide fragments which can bind to MHC class I antigen and which can be recognized by T cells in such binding state;

(2) DNA which comprises the base sequence shown in SEQ ID NO: 2, or a variant DNA which hybridizes to said DNA under stringent conditions, the protein produced by expression of said DNA and variant DNA being capable of yielding, through its intracellular decomposition, peptide fragments which can bind to MHC class I antigen and which can be recognized by T cells in such binding state;

(3) medicines comprising DNA of the above item (1) or (2) as an active ingredient;

(4) expression plasmids comprising DNA of the above item (1) or (2);

(5) transformants transformed with the expression plasmid of the above item (4);

(6) tumor antigen proteins produced by expression of DNA of the above item (1) or (2);

(7) tumor antigen peptides comprising part of the protein of the above item (6) which can bind to MHC class I antigen to be recognized by T cells, or derivatives thereof having functionally equivalent properties;

(8) tumor antigen peptides of the above item (7) which comprise all or part of the amino acid sequence of positions 749-757, 736-744, 785-793, or 690-698 in the amino acid sequence of SEQ ID NO: 1, or derivatives thereof having functionally equivalent properties;

(9) medicines comprising, as an active ingredient, tumor antigen protein

of the above item (6), tumor antigen peptide or derivative thereof defined in the above item (7) or (8).

(10) antibodies which specifically bind to the tumor antigen proteins of the above item (6) or tumor antigen peptides of the above item (7) or (8); and

(11) DNA comprising 8 or more bases having a sequence complementary to the coding or 5' non-coding sequence of DNA having the base sequence shown in SEQ ID NO: 2, or RNA corresponding to said DNA, or chemically modified variant thereof.

MODE FOR CARRYING OUT THE INVENTION

DNAs of the present invention encode a novel tumor antigen protein, and may include a DNA which encodes a protein having the amino acid sequence shown in SEQ ID NO: 1 or a variant protein thereof in which one or more amino acid residues are substituted, deleted or added, said protein and variant protein being capable of yielding, through its intracellular decomposition, peptide fragments which can bind to MHC class I antigen and which can be recognized by T cells in such binding state, as well as DNA which comprises the base sequence shown in SEQ ID NO: 2 or variant DNA thereof which hybridizes to said DNA under stringent conditions, the protein produced by expression of said DNA and variant DNA being capable of yielding, through its intracellular decomposition, peptide fragments which can bind to MHC class I antigen and which can be recognized by T cells in such binding state.

As used herein, the phrase "variant protein thereof in which

one or more amino acid residues are substituted, deleted, or added" refers to so-called variant proteins artificially prepared, to naturally-occurring polymorphism, or to proteins produced by mutation or modification but having functionally equivalent properties. DNAs encoding such variant proteins may be prepared using, for example, the methods described in Sambrook, J. *et al.*, *Molecular Cloning: A Laboratory Manual*, 2nd ed., vols. 1-3 (Cold Spring Harbor Laboratory Press, New York, 1989), such as site-directed mutagenesis or PCR method. In this context, the number of amino acid residues to be substituted, deleted, or added should be such a number that permits the substitution, deletion or addition by well-known methods such as site-directed mutagenesis described above.

"Variant DNA which hybridizes to DNA under stringent conditions" as described herein may be obtained using, for example, the methods described in *Molecular Cloning* mentioned above. In this context, "stringent conditions" refers to, for example, such conditions that hybridization is conducted at 42°C in a solution containing 6x SSC (20x SSC means 333 mM sodium citrate and 333 mM NaCl), 0.5% SDS, and 50% formamide, followed by washing in a solution of 0.1x SSC and 0.5% SDS at 68°C, or those conditions described in Nakayama *et al.*, *Bio-Jikken-Illustrated*, vol. 2, "Idenshi-Kaiseki-no-Kiso (Basis for Gene Analysis)", pp. 148-151, Shujunsha,.1995. For the purpose of this invention, the protein produced by expression of such hybridizable DNA should comprise a peptide segment which is capable of binding to MHC class I antigen and recognized by T cells.

As used herein, "protein and variant protein which are capable of yielding, through its intracellular decomposition, peptide fragments which can bind to MHC class I antigen and which can be recognized by T cells in such binding state" (hereinafter, such protein is sometimes referred to as tumor antigen protein) means that partial peptide consisting of part of the amino acid sequence of such protein or variant protein can bind to MHC class I antigen, and that when bound to MHC class I antigen and presented on cell surface, the complex of the peptide fragment and MHC class I antigen can be recognized by T cells capable of specifically binding thereto, and transduces signals to T cells. In this context, such binding means non-covalent binding.

In order to confirm that a given peptide fragment is capable of binding to MHC class I antigen and recognized by T cells, the peptide fragment may be bound to MHC class I antigen and presented on cell surface by expressing it endogenously in an appropriate cell or by adding it exogenously to an appropriate cell (pulsing). The peptide presenting cells may be then treated with T cells specific to the tumor antigen protein, and cytokines produced by the T cells may be measured. Alternatively, as a method measuring the cytotoxic activity of T cells against the peptide-presenting cells, a method using the peptide-presenting cells labeled with $^{51}Cr$ (*Int. J. Cancer*, 58:317 (1994)) may also be used. In such methods, CTLs are preferably used as the T cells recognizing the peptide.

DNA of the present invention may be used as an active ingredient of medicines. For example, medicines which comprise DNA

of the present invention as an active ingredient can be used for treating or preventing tumors by administering the DNA of the present invention to tumor patients. When DNA of the present invention is administered, the tumor antigen protein is expressed at high level in the cells. As a result, the tumor antigen peptides bind to MHC class I antigen and presented on the cell surface at high density. This will cause efficient proliferation of tumor-specific CTLs in the body, allowing treatment or prevention of the tumor. Administration and introduction of DNA of the present invention into cells may be achieved using viral vectors or according to any one of other procedures (*Nikkei-Science*, April, 1994, pp. 20-45; *Gekkan-Yakuji*, **36**(1), 23-48 (1994); *Jikken-Igaku-Zokan*, **12**(15), 1994, and references cited therein).

Examples of the methods using viral vectors include those methods in which DNA of the present invention is incorporated into DNA or RNA virus such as retrovirus, adenovirus, adeno-associated virus, herpesvirus, vaccinia virus, poxvirus, poliovirus, or Sindbis virus, and introduced into cells. Among them, the methods using retrovirus, adenovirus, adeno-associated virus, or vaccinia virus are particularly preferred.

Other methods may include those in which expression plasmids are directly injected intramuscularly (DNA vaccination), the liposome method, Lipofectin method, microinjection, the calcium phosphate method, and electroporation, with DNA vaccination and the liposome method being particularly preferred.

In order to make DNA of the present invention act as medicine

in practice, one can use either of two methods: *in vivo* method in which DNA is directly introduced into the body, or *ex vivo* method in which certain cells are removed from human, and after introducing DNA into said cells extracorporeally, reintroduced into the body (*Nikkei-Science*, April, 1994, pp. 20-45; *Gekkan-Yakuji*, **36**(1), 23-48 (1994); *Jikkenn-Igaku-Zokan*, **12**(15), 1994; and references cited therein). *In vivo* method is rather preferred.

In the case of *in vivo* methods, DNA may be administered by any appropriate route depending on the diseases and symptoms to be treated, and other factors. For example, it may be administered by intravenous, intraarterial, subcutaneous, intracutaneous, or intramuscular routes. In the case of *in vivo* methods, such medicines may be administered in various dosage forms such as solution, and they are typically formulated into injections containing DNA of the present invention as an active ingredient, which may also include, if necessary, conventional carriers. When DNA of the present invention is included in liposomes or membrane-fused liposomes (such as Sendai virus (HVJ)-liposomes), such medicines may be in the form of suspension, frozen drug, centrifugally-concentrated frozen drug or the like.

Although the amount of DNA of the present invention in such formulations may vary depending on, for example, the disease to be treated, the age and body weight of a particular patient, it is usually preferred to administer 0.0001-100 mg, more preferably 0.001-10 mg, of DNA of the present invention every several days to every several months.

Furthermore, the tumor antigen protein can be prepared in large quantities by recombinant DNA techniques using DNA of the present invention.

Preparation of tumor antigen protein by expression of DNA of the present invention may be achieved according to many publications and references such as *Molecular Cloning* mentioned above. An expression plasmid which can replicate and function in host cells is constructed by adding regulatory gene(s) such as a promoter which controlls transcription (e.g., trp, lac, T7, or SV40 early promoter) upstream to the DNA to be expressed and by inserting the resultant DNA into an appropriate vector (e.g., pSV-SPORT1). The expression plasmid is then introduced into appropriate host cells to obtain transformants. Examples of host cell include, for example, prokaryotes such as *Escherichia coli*, unicellular eukaryotes such as yeast, and cells derived from multicellular eukaryotes such as insects or animals. Gene transfer into host cells may be achieved by, for example, the calcium phosphate method, DEAE-dextran method, or the electric pulse method. Transformants cultured in appropriate medium produce the protein of interest. The tumor antigen protein thus obtained may be isolated and purified according to standard biochemical procedures.

In the present invention, "peptide fragments which can bind to MHC class I antigen and which can be recognized by T cells in such binding state", which may be produced through intracellular decomposition of tumor antigen protein of the present invention, *i.e.*, "tumor antigen peptides", may be determined as follows.

Firstly, fragments of DNA encoding tumor antigen protein and having various sizes are prepared using, for example, PCR, exonucleases, or restriction enzymes, and then inserted into expression vectors as described above. The vectors are then cotransfected into cells not expressing tumor antigen proteins (e.g., COS cells), with a plasmid which comprises a gene for MHC class I antigen that presents tumor antigens, in order to express them transiently. The regions which include the tumor antigen peptides are identified on the basis of the reactivity of the transfectants with CTL. Subsequently, various peptides included in such regions are synthesized. Cells expressing MHC class I antigen which presents tumor antigens but not expressing tumor antigen proteins are pulsed with the synthesized peptides, and examined for their reaction with CTL to identify the tumor antigen peptides (*J. Exp. Med.*, **176**:1453, 1992; *J. Exp. Med.*, **179**:24, 759, 1994).

Alternatively, the sequence regularities (motifs) of antigen peptides bound and presented by certain MHC types such as HLA-A1, -A0201, -A0205, -A11, -A24, -A31, -A6801, -B7, -B8, -B2705, -B37, -Cw0401, and -Cw0602 have been known, and threfore, candidates for tumor antigen peptides may also be selected making reference to such motifs, and such candidate peptides may be synthesized and identified in the manner as described above (*Eur. J. Immunol.*, **24**:759, 1994; *J. Exp. Med.*, **180**:347, 1994).

It is also known that MHC includes class II antigens besides class I antigens, and that conjugates of such MHC class II antigen with

particular tumor antigen peptides, which may be produced from tumor antigen protein through phagocytosis and decomposition by antigen-presenting cells, such as macrophage, will activate tumor-specific helper T cells (*J. Immunol.*, **146**:1708-1714, 1991).

The successful cloning of the novel tumor antigen protein gene of the present invention also enables those skilled in the art to determine additional tumor antigen peptides which bind to MHC class II antigen described above. Specifically, such antigen peptides may be determined on the basis of their reactivity with T cells or based on known information on motifs of such antigen peptides, in the same manner as MHC class I antigen.

The tumor antigen peptides thus determined may be prepared by usual methods known in peptide chemistry such as those described in "*Peptide Synthesis*" (Interscience, New York, 1966), "*The Proteins*" (vol. 2, Academic Press Inc., New York, 1976), "*Pepuchido-Gosei*" (Maruzen, 1975), or "*Pepuchido-Gosei-no-Kiso-to-Jikkenn*" (Maruzen, 1985). In particular, such peptide can be synthesized by selecting either the liquid phase method or the solid phase method depending on the structure of its C-terminus, with the liquid phase method being more preferable. Thus, peptides may be prepared by protecting and deprotecting functional groups in amino acids, and elongating them by a single residue or several residues. Protecting groups for functional groups on amino acids are described, for example, in the above-mentioned publications concerning peptide chemistry.

For the purpose of the present invention, "tumor antigen

peptides" may be defined as peptide fragments derived from either a protein having the amino acid sequence shown in SEQ ID NO: 1 or a variant protein thereof as defined above. Although the following description mainly relates to tumor antigen peptides derived from the protein having the amino acid sequence shown in SEQ ID NO: 1 as well as derivatives thereof, it will be understood that such description can apply to tumor antigen peptides derived from variant proteins.

Tumor antigen peptides produced by intracellular decomposition of the protein shown in SEQ ID NO: 1 are not specifically restricted, and may include, but not limited to, those peptides that comprise all or part of the amino acid sequence of positions 749-757, 736-744, 785-793, or 690-698 in the amino acid sequence shown in SEQ ID NO: 1. Preferred are those peptides that consist of 9 amino acid residues, and those peptides that consists of the amino acid sequence of positions 749-757, 736-744, 785-793, or 690-698 in SEQ ID NO: 1 are particularly preferred. Regarding tumor antigen peptides described herein, for example, the peptide consisting of the amino acid sequence of positions 749-757 in SEQ ID NO: 1 is sometimes abbreviated as "749-757".

As used herein, "derivatives of tumor antigen peptide" refers to those derivatives which have properties functionally equivalent to such tumor antigen peptide and in which some of the amino acid residues in said peptide are substituted, deleted, or added, or to those derivatives in which amino group(s) or carboxy group(s) in said peptide(s) or derivatives described just above are modified. In particular, examples

of such derivatives may include those derivatives in which, in a tumor antigen peptide of the present invention comprising all or part of the amino acid sequence of positions 749-757, 736-744, 785-793, or 690-698 in the amino acid sequence of SEQ ID NO: 1, some of the amino acid residues in the amino acid sequence of positions 749-757, 736-744, 785-793, or 690-698 are substituted or deleted, or other amino acid residue(s) are added thereto.

Among derivatives in which some of the amino acid residues in said peptide are substituted, deleted, or added, preferred are those derivatives which retain the epitope regions in the tumor antigen peptides involved in their binding with CTL and in which amino acid residue(s) in the tumor antigen peptides involved in their binding with MHC class I antigen are substituted, deleted, or added. Among such derivatives, those derivatives in which a single amino acid residue is substituted are more preferred (*Immunol.* **84**:298-303, 1995). For antigen peptides derived from melanoma tumor antigen protein gp 100, it is reported that substitution of amino acid(s) in the binding site for MHC class I antigen has resulted in its stronger binding with MHC class I antigen, and also caused stronger induction of CTL specific to such antigen peptide when used in *in vitro* stimulation of peripheral blood lymphocytes derived from melanoma patients (*J. Immunol.*, **157**:2539-2548, 1996).

Such derivatives can be easily synthesized using a commercially available peptide synthesizer, and the binding affinity of synthesized derivatives to MHC class I antigen may be easily measured

by competitive inhibition assay between said derivatives and radiolabeled standard peptide for binding to MHC class I antigen (R. T. Kubo *et al.*, *J. Immunol.*, **152**:3913, 1994). Thus, by subjecting various peptide derivatives to such assay, peptide derivatives having CTL-inducing activity can be easily selected. Since the peptide derivatives thus selected can bind to MHC class I antigen more strongly while retaining their binding ability to CTL, they can be used as more efficient tumor antigen peptides.

Examples of modifying group for amino group may include acyl groups, and in particular, alkanoyl groups of 1-6 carbon atoms, alkanoyl groups of 1-6 carbon atoms substituted by phenyl group, carbonyl groups substituted by cycloalkyl group of 5-7 carbon atoms, alkylsulfonyl groups of 1-6 carbon atoms, phenylsulfonyl groups, and the like.

Modifying group for carboxy group include, for example, ester and amide groups. Specific examples of such ester group may be alkyl ester groups of 1-6 carbon atoms, alkyl ester groups of 0-6 carbon atoms substituted by phenyl group, and cycloalkyl ester groups of 5-7 carbon atoms, and specific examples of such amide group may be an amide group, amide groups substituted by one or two alkyl groups of 1-6 carbon atoms, amide groups of 0-6 carbon atoms substituted by one or two alkyl groups substituted by phenyl, and amide groups forming a 5-7 membered azacycloalkane including the amide nitrogen as a ring member.

"Antibodies" of the present invention may be easily prepared according to, for example, the methods described in Lane, H.D. *et al.*,

*Antibodies: A Laboratory Manual* (Cold Spring Harbor Labortory Press, New York, 1989). Specifically, antibodies which recognize tumor antigen proteins or tumor antigen peptides, and antibodies which further neutralize their activities may be easily prepared by immunizing an animal with such tumor antigen protein or tumor antigen peptide using conventional procedures. Such antibodies may be used in, for example, affinity chromatography, screening of cDNA library, immunological diagnosis, or preparation of medicines. Such immunological diagnosis may include immunoblotting, radioimmunoassay (RIA), enzyme-linked immunosorbent assay (ELISA), fluorescent or luminescent assay, and the like.

As used herein, "DNA comprising 8 or more bases having a sequence complementary to the coding sequence or 5' non-coding sequence of DNA comprising the base sequence shown in SEQ ID NO: 2, or RNA corresponding to said DNA" means an antisense strand of double stranded DNA, or RNA corresponding to such antisense strand DNA, comprising 8 or more bases (hereinafter referred to as antisense oligonucleotides).

For example, as such antisense oligonucleotides, DNA may be prepared on the basis of the base sequence of the gene encoding tumor antigen protein of the present invention, and corresponding RNA may be prepared by incorporating such DNA into an expression plasmid in the antisense direction.

Although such antisense oligonucleotides may have a sequence complimentary to any part of the coding sequence or 5' non-coding

sequence of DNA of the present invention comprising the base sequence shown in SEQ ID NO: 2, they preferably have a sequence complimentary to transcription initiation site, translation initiation site, 5' non-translated region, a boundary region between exon and intron, or 5' CAP region.

In the above description, "chemically modified variants" of "DNA or RNA corresponding to said DNA" (hereinafter referred to as chemically modified variant of antisense oligonucleotides) may include those variants which have increased transferability into cells or increased stability in cells. Specific examples of the variants include phosphorothioate, phosphorodithioate, alkyl phosphotriester, alkyl phosphonate, or alkyl phosphoamidate derivatives ("*Antisense RNA and DNA*", WILLEY-LISS, 1992, pp. 1-50). Such chemically modified variant may be prepared according to, for example, the above-mentioned reference.

Such antisense oligonucleotides or chemically modified variants thereof may be used to control expression of the gene encoding tumor antigen protein. Since such control can decrease the amount of tumor antigen protein to be produced, and thereby decrease a damage caused by CTLs and also inhibit proliferation of CTL, autoimmune diseases due to over-expression of tumor antigen protein may be treated or prevented by such approach.

When the antisense oligonucleotides or chemically modified variants thereof are administered as such, preferred length thereof may be 8-200 bases, more preferably 10-25 bases, and most preferably 12-25

bases.

When inserted into expression plasmids, preferred length of the antisense oligonucleotides may be 100 bases or more, preferably 300-1000 bases, and more preferably 500-1000 bases.

Antisense oligonucleotides inserted in expression plasmids may be introduced into cells according to, for example, the methods described in *Jikken-Igaku*, vol. 12 (1994), such as those employing liposomes or recombinant viruses. Expression plasmids for antisense oligonucleotides may be easily prepared using conventional expression vectors just by placing the genes of the present invention after the promoter in the opposite direction so that the genes of the present invention may be transcribed in the direction from 3' to 5'.

When administered as such, antisense oligonucleotides or chemical variants of the antisense oligonucleotides may be formulated by mixing them with stabilizing agents, buffers, solvents, and/or the like, and then administered simultaneously with antibiotics, anti-inflammatory agents, or anesthetics. The formulations thus prepared may be administered via various routes. Such formulations are preferably administered everyday or every several days to every several weeks. Furthermore, in order to avoid such frequent administration, sustained-release minipellet formulation may also be prepared and implanted near the affected area. Alternatively, the formulation may be slowly administered in continuous manner using, for example, an osmotic pump. Dosage are typically to be adjusted so that the concentration at the site of action will be from 0.1 nM to 10 μM.

Tumor antigen proteins, tumor antigen peptides, and derivatives thereof having functionally equivalent properties, of the present invention may be used alone or in combination, and medicines comprising them as an active ingredient may be administered together with adjuvants or in particulate dosage form in order to effectively establish the cellular immunity. Specifically, when tumor antigen protein or tumor antigen peptide is administered to a subject, tumor antigen peptides are presented at high density on MHC class I antigens of the antigen-presenting cells, resulting in efficient proliferation of tumor-specific CTLs. For such purpose, those adjuvants described in the literature (*Clin. Microbiol. Rev.*, 7:277-289, 1994) are applicable. The active ingredient(s) are administered in a dosage form which allows the foreign antigen peptide to be efficiently presented on MHC class I antigen, such as liposomal preparations, particulate preparations in which the active ingredient(s) are bound to beads having a diameter of several μm, or preparations in which the active ingredient(s) are bound to lipids. It may be also possible to administer antigen-presenting cells such as dendritic cells or macrophages pulsed with the tumor antigen peptide, or cells transfected with DNA encoding the tumor antigen protein. Although the dose of the tumor antigen protein or tumor antigen peptide of the present invention in such preparations may be appropriately adjusted depending on various factors such as the disease to be treated, age and body weight of a particular patient, preferred dose is between 0.0001 mg and 1000 mg, and more preferably between 0.001 mg and 1000 mg. It is preferably administered every several days to

every several months.

A method for *in vitro* induction of CTL from peripheral lymphocytes using tumor antigen peptide of the present invention is exemplified as follows.

Peripheral blood lymphocytes from an esophageal cancer patient with squamous cell carcinoma are *in vitro*-cultured, and a tumor antigen peptide of the present invention, for example, a peptide having the sequence of "736-744", "749-757", "785-793", or "690-698" is added to the culture medium at the final concentration of 10 μg/ml, in order to stimulate the peripheral blood lymphocytes. Such stimulation is repeated three times at intervals of one week. One week after the third stimulation, the peripheral blood lymphocytes are recovered, and measured for their cytotoxic activity according to the methods described in D. D. Kharkevitch et al, Int. J. Cancer, 58:317 (1994), in order to find CTL-inducing activity of the tumor antigen peptide of the present invention.

The method of the present invention for diagnosing tumors or autoimmune diseases may be conducted using antibodies specifically binding to a tumor antigen protein or tumor antigen peptide. Examples of such method may include those detecting tumor antigen protein in tumor tissue preparations, or detecting the presence of tumor antigen protein or antibodies against tumor antigen protein in blood or tissues. Such detection may be achieved by any appropriate method selected from, for example, immunohistochemical methods, immunoblotting, radioimmunoassay (RIA), enzyme-linked immunosorbent assay (ELISA),

fluorescent and luminescent assays. Furthermore, detection of tumor antigen protein using antibodies enables early detection of tumors or their recurrence, as well as selection of patients who may be suitably treated with the tumor antigen proteins, tumor antigen peptides, or DNA encoding them.

BRIEF DESCRIPTION OF DRAWINGS

Fig. 1 is electrophoretograms showing the result of Northern blot hybridization described in Example 2.

In Fig. 1 a), KE-4, KE-3, TE-8, and TE-9 indicate esophageal cancer cell lines; Kuma-1 indicates a head and neck cancer cell line; HSC-4 indicates a mouth cancer cell line; Bec-1 indicates a B cell line; KMG-A indicates a gallbladder cancer cell line; R-27 indicates a breast cancer cell line; KIM-1, KYN-1, and HAK-3 indicate hepatic cancer cell lines; and M36 and M37 indicate melanoma cell lines.

EXAMPLES

The following detailed examples are presented by way of illustration of certain specific embodiments of the invention. The Examples are representative only and should not be construed as limiting in any respect.

Reference Example 1

Establishment of Cytotoxic T Lymphocyte (CTL) Cell Line against Esophageal Cancer Cell Line

According to the disclosure of Nakao *et al.*, *Cancer Res.*, 55:4248-4252 (1995), CTL against an esophageal cancer cell line, KE-4, belonging to squamous cell carcinomas when classified on the basis of

the tissue type was established from peripheral blood monocytes of a patient, named KE-4CTL, and used in experiments. The esophageal cancer cell line KE-4 and KE-4CTL have been deposited at The National Institute of Bioscience and Human Technology (1-1-3 Higashi, Tsukuba, Ibaraki, Japan) under International Deposition Nos. FERM BP-5955 and FERM BP-5954, respectively, both on May 23, 1997. Furthermore, typing of HLA class I molecules of KE-4 was conducted according to the above-noted disclosure of Nakao *et al.*, and it was confirmed that they are HLA-A2402, -A2601, B54, -B60, -Cw1, and -Cw3.

Reference Example 2

Preparation of HLA-A2061 cDNA and HLA-A2402 cDNA

Using KE-4, a recombinant plasmid was prepared by incorporating cDNA for HLA-A2601 into an expression vector pCR3 (INVITROGEN) according to the disclosure of Nakao *et al.*, *Cancer Res.*, **55**:4248-4252 (1995). Another recombinant plasmid for HLA-A2402 was also prepared in the similar manner.

Reference Example 3

Preparation of cDNA Library derived from KE-4

Poly $(A)^+$ mRNA was prepared from KE-4 by isolation of total RNA fraction and purification on oligo (dT) column using mRNA Purification system (manufactured by Pharmacia Biotech) according to the manufacturer's protocol. cDNAs having *Not* I adapter and *Sca* I adapter linked to each terminus were prepared from mRNAs using SuperScript™ Plasmid System (Gibco BRL) according to the manufacturer's protocol, and then ligated to an expression vector,

plasmid pSV-SPORT1 (Gibco BRL), digested with restriction enzymes *Not* I and *Sal* I, to yield recombinant plasmids. The recombinant plasmids were introduced into E. coli. ElectroMAX DH10B/p3™ cells (Gibco BRL) using electric pulses in Gene Pulser (Bio-Rad) under conditions of 25 μF and 2.5 kV. Transformants into which the recombinant plasmids had been introduced were selected in LB medium (1% bacto-trypton, 0.5% yeast extract, 0.5% NaCl, pH7.3) containing ampicillin (50 μg/ml).

Reference Example 4

Quantitative Determination of Interferon-γ

Quantitative Determination of interferon-γ (IFN-γ) was conducted by enzyme immunoassay (ELISA). Anti-human IFN-γ mouse monoclonal antibody as a solid-phased antibody was adsorbed on wells of 96-well microplate, and after blocking non-specific bindings with bovine serum albumin, allowed to bind with IFN-γ in samples. Anti-human IFN-γ rabbit polyclonal antibody as a detection antibody was then allowed to bind, and after binding with an anti-rabbit immunoglobulin goat antibody labeled with alkaline phosphatase, reacted with para-nitrophenyl phosphate as a chromogenic substrate. After stopping the reaction by adding an equal volume of 1N NaOH, absorbance at 405 nm was measured. The absorbance was compared with that obtained with standard IFN-γ to determine the amount of IFN-γ in the sample.

Example 1

Screening of Gene for Novel Tumor Antigen Protein

The recombinant plasmid DNAs were recovered from pools of about 100 transformants described in Reference Example 3 as follows. A hundred transformants were introduced and cultured in each well of 96-well U-bottomed microplate containing LB medium plus ampicillin (50 μg/ml). Part of the culture was then transferred to another 96-well U-bottomed microplate containing 0.25 ml per well of TYGPN medium (F.M. Ausubel *et al.*, *Current Protocols in Molecular Biology*, John Wiley & Sons, Inc.), and cultured for 48 hours at 37°C. The remaining cultures in LB medium on the microplate were stored in frozen. Preparation of recombinant plasmid DNAs from transformants cultured in TYGPN medium was achieved in the microplate by alkaline lysis (F.M. Ausubel *et al.*, *Current Protocols in Molecular Biology*, John Wiley & Sons, Inc.). The recombinant plasmid DNAs recovered by isopropanol precipitation were suspended in 50 μl of 10 mM Tris, 1 mM EDTA, pH 7.4, containing 20 ng/ml RNase.

Fibroblast cell line, VA-13 cells (RIKEN CELL BANK, The Institute of Physical and Chemical Research; *Ann. Med. Exp. Biol. Fenn.*, **44**:242-254, 1966) were doubly transfected with the recombinant plasmid for KE-4 cDNA and the recombinant plasmid for HLA-A2601 cDNA using Lipofectin method as follows. Seven thousands VA-13 cells were placed in each well of 96-well flat-bottomed microplate, and incubated for 2 days in 100 μl of RPMI 1640 medium containing 10% FCS. Using Lipofectin reagent (Gibco BRL), 30μl of 70 μl mixture consisting of 25 μl of the recombinant plasmid for KE-4 cDNA

corresponding to about 100 transformants, 10 μl (200 ng) of the recombinant plasmid for HLA-A2601 cDNA described in Reference Example 2, and 35 μl of about 35-fold diluted Lipofectin reagent was added to VA-13 cells to be doubly transfected. Transfectants were prepared in duplicate. After 5 hours, 200 μl of culture medium containing 10% FCS was added to the transfectants, and further incubated for 72 hours at 37°C. After removing the culture medium, 10,000 KE-4CTL cells were added to each well, and cultured for 24 hours at 37°C in 100 μl of culture medium containing 10% FCS and 25 U/ml IL-2. The culture medium was recovered, and measured for IFN-γ by ELISA.

Regarding four groups in which high production of IFN-γ was observed, corresponding frozen-stored pools of about 100 transformants containing recombinant plasmids for KE-4 cDNA were used in the following screening. The pools of the transformants were plated on LB agar medium containing ampicillin (50 μg/ml) to obtain colonies. Two hundreds colonies for each group (total 800 colonies) were cultured as described above so that a single kind of transformant is included in each well, thereby recombinant plasmid DNAs for KE-4 cDNA were prepared. Then, VA-13 cells were doubly transfected with the recombinant plasmid for KE-4 cDNA and the recombinant plasmid for HLA-A2601 cDNA followed by cocultivation with KE-4CTL, and IFN-γ produced due to KE-4CTL reaction was quantitatively determined as described above in order to select positive plasmids. In this manner, a single KE-4

cDNA recombinant plasmid clone was selected and named 6DI. Furthermore, similar procedures were repeated with 6DI to determine the amount of IFN-γ produced by KE-4CTL according to the method described in Reference Example 4. The results are shown in the following TABLE 1.

TABLE 1

| Target cell | Amount of IFN-γ produced by KE-4CTL (pg/ml) |
|---|---|
| VA-13 cell | 0 |
| VA-13 cell + HLA-A2601 | 1.8 |
| VA-13 cell + 6DI | 4.3 |
| VA-13 cell + HLA-A2601 + 6DI | 24.0 |
| VA-13 cell + HLA-A0201[1)] | 0.9 |
| VA-13 cell + HLA-A0201 + 6DI[1)] | 3.0 |

[1)] For comparison, HLA of different type was transfected. (These date was obtained by transfection using the following amounts of DNA: 200 ng of HLA-A2601 or HLA-A0201, 100 ng of 6DI.)

Example 2

Expression Analysis for Tumor Antigen Protein Gene by Northern Hybridization

RNAs were prepared from various cell lines using RNAzol B (TEL-TEST, Inc.). Five μg of RNA was denatured in the presence of formamide and formaldehyde, electrophoresed on agarose, then transferred and fixed onto Hybond-N+ Nylon membrane (Amersham). As RNAs from normal tissues, commercially available membranes (Clontech) onto which mRNAs have been preblotted were used. The

inserted sequence region of the recombinant plasmid 6DI cloned in Example 1 was labeled with $^{32}P$ using Multiprime DNA labelling system (Amersham) to prepare DNA probe. According to the known method (Nakayama *et al.*, *Bio-Jikken-Illustrated*, vol. 2, "Idenshi-Kaiseki-No-Kiso (A Basis for Gene Analysis)", pp. 148-151, Shujunsha, 1995), this probe was hybridized to RNAs on the membranes, and subjected to autoradiography to detect mRNA for tumor antigen protein gene of the present invention. The membranes used for the detection of mRNA for said gene were boiled in 0.5% aqueous sodium dodecyl sulfate to remove the probe, and subjected to Northern hybridization in a similar manner using β-actin as a probe which is constitutively expressed in cells, in order to detect mRNA which was used as an internal standard. The results are shown in Fig. 1. It became apparent from these results that mRNA for tumor antigen protein gene of the present invention is widely expressed in various cancer cells and normal tissues, and is about 2.5 kb in full length (Fig. 1).

Example 3

Cloning and Base Sequencing of Full-Length cDNA Clone Encoding Tumor Antigen Protein

KE-4-derived cDNA Library described in Reference Example 3 was plated on LB agar medium containing ampicillin (50μg/ml). The colonies thus obtained were then transferred and fixed on Hybond-N+ nylon membrane (Amersham) according to the manufacturer's protocol. The same 6DI probe as that used in Example 2 was employed for hybridization and autoradiography under the same conditions as those

used in Example 2, in order to select colonies which contain recombinant plasmids having the cDNA for tumor antigen protein gene incorporated. Furthermore, recombinant plasmids were recovered from the colonies selected, treated with restriction enzymes *Not* I and *Sal* I, and then electrophoresed on agarose to determine the length of incorporated cDNAs. A recombinant plasmid incorporating cDNA of about 2.5 kb was selected, and named K3. The base sequence of the cDNA region in this plasmid K3 was determined using DyeDeoxy Terminator Cycle Sequencing kit (Perkin-Elmer). The base sequence thus determined is shown in SEQ ID NO: 2. The full-length of the cDNA was 2527 base pairs. The amino acid sequence (800 amino acids) encoded by the base sequence of SEQ ID NO: 2 is shown in SEQ ID NO: 1.

The analysis indicated that the base sequence shown in SEQ ID NO: 2 does not show homology with known tumor antigen protein genes derived from melanomas and thus proved to be a different gene. The search for the base sequence of SEQ ID NO: 2 using WWW Entrez database revealed that part of the base sequence of the present invention exhibits high homology more than 90% to three gene sequences, functions of which are not known, decoded by WashU-Merck EST Project and registered at GENBANK under Accession Nos. R89163, R62890, and R00027. No. R89163 corresponds to the sequence of positions 1893-2267; R62890 corresponds to the sequence of positions 2018-2389; and R00027 corresponds to the sequence of positions 2024-2510. These sequences correspond, however, to the base sequences 3' to the initiation codon in the base sequence of the present invention, and

therefore, the amino acid sequences they encode can not be determined.

After determination of the base sequence as described above, the plasmid K3 was introduced into *E. coli* JM109 to obtain *E. coli* JM109(K3) which is a transformant for storage containing the novel tumor antigen protein cDNA of the present invention. *E. coli* JM109(K3) has been deposited at The National Institute of Bioscience and Human Technology (1-1-3 Higashi, Tsukuba, Ibaraki, Japan) under International Deposition No. FERM BP-5951 on May 22, 1997.

Furthermore, cDNA library derived from normal human tissue (peripheral blood lymphocyte) was also screened in the manner as described above. This screening resulted in cloning of a recombinant plasmid into which cDNA of about 2.5 kb has been incorporated. It was found by determining the base sequence of this cDNA that cDNA thus cloned was the same as that shown in SEQ ID NO: 2 except for position 812 (position 812 for normal human tissue was T). It was thus indicated that in connection with the full-length gene comprising the gene encoding the tumor antigen protein of the present invention, almost the same genes are expressed in both cancer cells and normal human tissue.

VA-13 Cells were then doubly transfected with the recombinant plasmid K3 incorporating cDNA for the novel tumor antigen protein gene and another recombinant plasmid incorporating cDNA for HLA-A2601, and used as target cells. The amount of IFN-γ produced by the reaction of KE-4CTL was determined according to the method described in Reference Example 4. The results are shown in the

following TABLE 2.

TABLE 2

| Target cell | Amount of IFN-γ produced by KE-4CTL[1)] (pg/ml) |
|---|---|
| VA-13 cell + HLA-A2601 + K3 | 1439 |
| VA-13 cell + HLA-A0201[2)] + K3 | 10 |

[1)] Values obtained by subtracting the amount (background) of IFN-γ produced by KE-4CTL in response to VA-13 cells transfected with each HLA.

[2)] For comparison, HLA of different type was transfected.

(These date was obtained by transfection of the following amounts of DNA: 200 ng of HLA-A2601 or HLA-A0201, 100 ng of K3.)

Example 4

Identification of Tumor Antigen Peptide

From the recombinant plasmid 6DI cloned in Example 1 which incorporated partial cDNA for the novel tumor antigen protein gene, plasmids containing partial cDNA for tumor antigen protein gene of various length were prepared using Deletion Kit for Kilo-Sequence (Takara Shuzo Co.) according to the manufacturer's protocol. These plasmids were introduced into *E. coli* ElectroMax DH10B/p3™ cells (Gibco BRL). The cells were plated on agar medium, and 50 colonies were selected at random. From the colonies, plasmid DNAs were prepared, subjected to electrophoresis, and 5 clones which contained plasmids having appropriate length selected.

According to the method described in Example 1, VA-13 cells were doubly transfected with HLA-A2601 cDNA and the above plasmid DNA, cocultured with KE-4CTL, and IFN-γ in the culture medium was quantitatively determined according to the method described in Reference Example 4. As a result, it was found that the transfectant with a plasmid lacking the base sequence after position 2253 in SEQ ID No:2 had no IFN-γ-inducing activity on KE-4CTL. It was therefore suggested that peptides having the sequence after about position 739 in the amino acid sequence of SEQ ID NO: 1 may have IFN-γ-inducing activity on KE-4CTL.

Thus, a series of 21 different peptides each consisting of successive 10 amino acid residues in the amino acid sequence after position 730 in SEQ ID NO: 1 were synthesized so that they each have the amino acid sequence shifted serially by three amino acid residues. Using these peptides, IFN-γ in culture medium was determined as described above except that the antigen presentation was achieved by pulsing HLA-A2601 cDNA-transfected VA-13 cells with the peptides. As the result, IFN-γ-inducing activity was observed in the peptides having the amino acid sequences of "736-745", "748-757", and "784-793" in SEQ ID NO: 1.

For each of these three peptides, additional peptides consisting of 9 amino acid residues were synthesized by truncating the N- or C-terminal residue, and used for measurement of IFN-γ-inducing activity in a similar manner. Stronger IFN-γ-inducing activity was

observed for the peptides having the amino acid sequences of "736-744", "749-757", and "785-793" in SEQ ID NO: 1. The results are shown in TABLE 3.

TABLE 3

| Pulsed cell | Peptide | Amount of INF-γ produced by KE4-CTL cells (pg/ml) |
|---|---|---|
| VA-13/A2601[1)] | "736-744" | 203 |
| VA-13/A0201[2)] | "736-744" | 44 |
| VA-13/A2601 | "749-757" | 183 |
| VA-13/A0201 | "749-757" | 89 |
| VA-13/A2601 | "785-793" | 394 |
| VA-13/A0201 | "785-793" | 102 |

[1)] VA-13 cells transfected with HLA-A2601 cDNA

[2)] VA-13 cells transfected with different HLA-A0201 cDNA as a control

The results in TABLE 3 suggest that these peptides function as a tumor antigen peptides.

In addition, it is known that there are certain rules (motifs) in the sequences of antigen peptides bound and presented by HLA molecules. Concerning the motif for HLA-A24, the second amino acid is tyrosine and the ninth amino acid is isoleucine, leucine or phenylalanine in the sequence of antigen peptides consisting of 9 amino acid residues (*Immunogenetics*, **41**:178-228, 1995).

Thus, another peptide having the amino acid sequence of "690-698" in SEQ ID NO: 1 which corresponds to the above motif was further synthesized. VA-13 cells transfected with HLA-A2402 cDNA

was then pulsed with the peptide, and IFN-γ-inducing activity on KE-4CTL was measured as described above. The results are shown in TABLE 4

| Pulsed cell | Peptide | Amount of INF-γ produced by KE4-CTL cells (pg/ml) |
|---|---|---|
| VA-13 | "690-698" | 157 |
| VA-13/A2402[1)] | "690-698" | 269 |
| VA-13/A0201[2)] | "690-698" | 166 |

[1)] VA-13 cells transfected with HLA-A2402 cDNA

[2)] VA-13 cells transfected with different HLA-A0201 cDNA as a control

The results in TABLE 4 suggest that the peptide "690-698" functions as a tumor antigen peptide.

Example 5

Inducement of CTL from peripheral blood lymphocytes by tumor antigen peptides

The inventors have investigated whether antigen-specific CTL can be induced from peripheral blood lymphocytes of the cancer patient from whom KE-4 was derived, by *in vitro* stimulation with the tumor antigen peptides described in Example 3. Tumor antigen peptides used were those peptides having the sequences of "736-744", "749-757", and "690-698", obtained in the above Example 3. Frozen peripheral blood lymphocytes, which had been separated from the above cancer patient using Ficoll method, were awoke, transferred to 24-well plate at about $2x10^6$ cells/well, and cultured in RPMI 1640 medium containing 10% FCS and IL-2 (100 U/ml). To stimulate the peripheral blood

lymphocytes, the above tumor antigen peptide was added to the culture medium at 10 μg/ml. After one week, 10 μg/ml of the above tumor antigen peptide was added together with about $1x10^5$ cells of X ray-radiated (50 Gy) peripheral blood lymphocytes for the second stimulation. After additional one week, the third stimulation was conducted in a similar manner.

For peptides having the sequences of "736-744" and "749-757", peripheral blood lymphocytes were recovered one week after the third stimulation, and measured for their cytotoxic activity using, as target cells, $^{51}Cr$-labeled KE-4 and another esophageal cancer cell line KE-3 of which HLA-A loci are A2402 and A2, according to the method described in D.D. Kharkevitch *et al.*, *Int. J. Cancer*, **58**:317 (1994). The results are shown in TABLE 5.

TABLE 5

| Effector cell | Target cell | Toxic activity (%) |
|---|---|---|
| Peripheral blood lymphocytes stimulated with "736-744" | KE-4 | 22.1 |
| | KE-3 | 3.7 |
| Peripheral blood lymphocytes stimulated with "749-757" | KE-4 | 35.9 |
| | KE-3 | 24.2 |

When stimulated with the peptide having the sequence of "736-744", KE-4 was severely injured, whereas the negative control KE-3 was not injured. It was therefore demonstrated that CTL specific for KE-4 was induced. Similarly, when stimulated with the peptide having the sequence of "749-757", stronger cytotoxic activity was observed on KE-4, although nonspecific cytotoxic activity was also

observed on KE-3, suggesting that CTL specific for KE-4 was induced.

For peptide having the sequence of "690-698", peripheral blood lymphocytes were recovered after the third stimulation, and further cultured in RPMI-1640 medium containing 10% FCS, 50% AIM-V (Gibco BRL), and IL-2 (100 U/ml). Then, the cytotoxic activity was measured as above using $^{51}$Cr-labeled KE-4 and VA-13 cells as target cells. In addition, lymphocytes were isolated from peripheral blood of a normal individual of which HLA-A loci were homozygous A24, and measured for their cytotoxic activity in the same manner as above using, as target cells, $^{51}$Cr-labeled KE-4 and lung cancer cell line QG-56 of which HLA-A loci are homozygous A2601. The results are shown in TABLE 6.

TABLE 6

| Effector cell | Target cell | Toxic activity (%) |
|---|---|---|
| "690-698"-Stimulated peripheral blood lymphocytes from a cancer patient | KE-4 | 24.7 |
| | VA-13 | 13.8 |
| "690-698"-Stimulated peripheral blood lymphocytes from a normal individual | KE-4 | 17.7 |
| | QG-56 | 11.5 |

By stimulating peripheral blood lymphocytes from a cancer patient and from a normal individual with the peptide having the sequence of "690-698", stronger cytotoxic activity was observed on KE-4, although nonspecific cytotoxic activity was also observed on the negative controls VA-13 and QG-56 cells. The above results suggest that CTLs specific for KE-4 were induced.

## EFFECTS OF THE INVENTION

According to the present invention, there are provided medicines for activating antitumor immunity by means of tumor antigen proteins and tumor antigen peptides, medicines for treating autoimmune diseases, and medicines comprising DNA or the like encoding tumor antigen protein, as well as methods for diagnosing tumors or autoimmune diseases.

# SEQUENCE LISTING

SEQ ID NO: 1

SEQUENCE LENGTH: 800 amino acids

SEQUENCE TYPE: amino acid

TOPOLOGY: linear

MOLECULE TYPE: peptide

ORIGINAL SOURCE:

ORGANISM: human (*Homo sapiens*)

TISSUE TYPE: esophageal carcinoma tissue

FEATURE:

FEATURE KEY: peptide

LOCATION: 1..800

IDENTIFICATION METHOD: P

SEQUENCE DESCRIPTION:

Met Gly Ser Ser Lys Lys His Arg Gly Glu Lys Glu Ala Ala Gly Thr
5 10 15

Thr Ala Ala Ala Gly Thr Gly Gly Ala Thr Glu Gln Pro Pro Arg His
20 25 30

Arg Glu His Lys Lys His Lys His Arg Ser Gly Gly Ser Gly Gly Ser
35 40 45

Gly Gly Glu Arg Arg Lys Arg Ser Arg Glu Arg Gly Gly Glu Arg Gly
50 55 60

Ser Gly Arg Arg Gly Ala Glu Ala Glu Ala Arg Ser Ser Thr His Gly
65 70 75 80

Arg Glu Arg Ser Gln Ala Glu Pro Ser Glu Arg Arg Val Lys Arg Glu

85 90 95

Lys Arg Asp Asp Gly Tyr Glu Ala Ala Ala Ser Ser Lys Thr Ser Ser

100 105 110

Gly Asp Ala Ser Ser Leu Ser Ile Glu Glu Thr Asn Lys Leu Arg Ala

115 120 125

Lys Leu Gly Leu Lys Pro Leu Glu Val Asn Ala Ile Lys Lys Glu Ala

130 135 140

Gly Thr Lys Glu Glu Pro Val Thr Ala Asp Val Ile Asn Pro Met Ala

145 150 155 160

Leu Arg Gln Arg Glu Glu Leu Arg Glu Lys Leu Ala Ala Ala Lys Glu

165 170 175

Lys Arg Leu Leu Asn Gln Lys Leu Gly Lys Ile Lys Thr Leu Gly Glu

180 185 190

Asp Asp Pro Trp Leu Asp Asp Thr Ala Ala Trp Ile Glu Arg Ser Arg

195 200 205

Gln Leu Gln Lys Glu Lys Asp Leu Ala Glu Lys Arg Ala Lys Leu Leu

210 215 220

Glu Glu Met Asp Gln Glu Phe Gly Val Ser Thr Leu Val Glu Glu Glu

225 230 235 240

Phe Gly Gln Arg Arg Gln Asp Leu Tyr Ser Ala Arg Asp Leu Gln Gly

245 250 255

Leu Thr Val Glu His Ala Ile Asp Ser Phe Arg Glu Gly Glu Thr Met

260 265 270

Ile Leu Thr Leu Lys Asp Lys Gly Val Leu Gln Glu Glu Glu Asp Val

275 280 285

Leu Val Asn Val Asn Leu Val Asp Lys Glu Arg Ala Glu Lys Asn Val

290 295 300

Glu Leu Arg Lys Lys Lys Pro Asp Tyr Leu Pro Tyr Ala Glu Asp Glu
305 310 315 320
Ser Val Asp Asp Leu Ala Gln Gln Lys Pro Arg Ser Ile Leu Ser Lys
325 330 335
Tyr Asp Glu Glu Leu Glu Gly Glu Arg Pro His Ser Phe Arg Leu Glu
340 345 350
Gln Gly Gly Thr Ala Asp Gly Leu Arg Glu Arg Glu Leu Glu Glu Ile
355 360 365
Arg Ala Lys Leu Arg Leu Gln Ala Gln Ser Leu Ser Thr Val Gly Pro
370 375 380
Arg Leu Ala Ser Glu Tyr Leu Thr Pro Glu Glu Met Val Thr Phe Lys
385 390 395 400
Lys Thr Lys Arg Arg Val Lys Lys Ile Arg Lys Lys Glu Lys Glu Val
405 410 415
Val Val Arg Ala Asp Asp Leu Leu Pro Leu Gly Asp Gln Thr Gln Asp
420 425 430
Gly Asp Phe Gly Ser Arg Leu Arg Gly Arg Gly Arg Arg Arg Val Ser
435 440 445
Glu Val Glu Glu Glu Lys Glu Pro Val Pro Gln Pro Leu Pro Ser Asp
450 455 460
Asp Thr Arg Val Glu Asn Met Asp Ile Ser Asp Glu Glu Glu Gly Gly
465 470 475 480
Ala Pro Pro Pro Gly Ser Pro Gln Val Leu Glu Glu Asp Glu Ala Glu
485 490 495
Leu Glu Leu Gln Lys Gln Leu Glu Lys Gly Arg Arg Leu Arg Gln Leu
500 505 510
Gln Gln Leu Gln Gln Leu Arg Asp Ser Gly Glu Lys Val Val Glu Ile

515 520 525

Val Lys Lys Leu Glu Ser Arg Gln Arg Gly Trp Glu Glu Asp Glu Asp

530 535 540

Pro Glu Arg Lys Gly Ala Ile Val Phe Asn Ala Thr Ser Glu Phe Cys

545 550 555 560

Arg Thr Leu Gly Glu Ile Pro Thr Tyr Gly Leu Ala Gly Asn Arg Glu

565 570 575

Glu Gln Glu Glu Leu Met Asp Phe Glu Arg Asp Glu Glu Arg Ser Ala

580 585 590

Asn Gly Gly Ser Glu Ser Asp Gly Glu Glu Asn Ile Gly Trp Ser Thr

595 600 605

Val Asn Leu Asp Glu Glu Lys Gln Gln Gln Asp Phe Ser Ala Ser Ser

610 615 620

Thr Thr Ile Leu Asp Glu Glu Pro Ile Val Asn Arg Gly Leu Ala Ala

625 630 635 640

Ala Leu Leu Leu Cys Gln Asn Lys Gly Leu Leu Glu Thr Thr Val Gln

645 650 655

Lys Val Ala Arg Val Lys Ala Pro Asn Lys Ser Leu Pro Ser Ala Val

660 665 670

Tyr Cys Ile Glu Asp Lys Met Ala Ile Asp Asp Lys Tyr Ser Arg Arg

675 680 685

Glu Glu Tyr Arg Gly Phe Thr Gln Asp Phe Lys Glu Lys Asp Gly Tyr

690 695 700

Lys Pro Asp Val Lys Ile Glu Tyr Val Asp Glu Thr Gly Arg Lys Leu

705 710 715 720

Thr Pro Lys Glu Ala Phe Arg Gln Leu Ser His Arg Phe His Gly Lys

725 730 735

Gly Ser Gly Lys Met Lys Thr Glu Arg Arg Met Lys Lys Leu Asp Glu
740 745 750

Glu Ala Leu Leu Lys Lys Met Ser Ser Ser Asp Thr Pro Leu Gly Thr
755 760 765

Val Ala Leu Leu Gln Glu Lys Gln Lys Ala Gln Lys Thr Pro Tyr Ile
770 775 780

Val Leu Ser Gly Ser Gly Lys Ser Met Asn Ala Asn Thr Ile Thr Lys
785 790 795 800

SEQ ID NO: 2

SEQUENCE LENGTH: 2527 base pairs

SEQUENCE TYPE: nucleic acid

STRANDEDNESS: double

TOPOLOGY: linear

MOLECULE TYPE: cDNA to mRNA

HYPOTHETICAL: No

ANTI-SENSE: No

ORIGINAL SOURCE:

ORGANISM: human (*Homo sapiens*)

TISSUE TYPE: esophageal carcinoma tissue

FEATURE:

FEATURE KEY: 5' UTR

LOCATION: 1..38

IDENTIFICATION METHOD: E

FEATURE KEY: CDS

LOCATION: 39..2438

IDENTIFICATION METHOD: E

FEATURE KEY: 3' UTR

LOCATION: 2439..2506

IDENTIFICATION METHOD: E

FEATURE KEY: poly A site

LOCATION: 2507..2527

IDENTIFICATION METHOD: E

SEQUENCE DESCRIPTION:

GGTTCGGCGG CAGCCGGGCT CGGAGTGGAC GTGCCACTAT GGGGTCGTCC AAGAAGCATC 60

GCGGAGAGAA GGAGGCGGCC GGGACGACGG CGGCGGCCGG CACCGGGGGT GCCACCGAGC 120

AGCCGCCGCG GCACCGGGAA CACAAAAAAC ACAAGCACCG GAGTGGCGGC AGTGGCGGTA 180

GCGGTGGCGA ACGACGGAAG CGGAGCCGGG AACGTGGGGG CGAGCGCGGG AGCGGGCGGC 240

GCGGGGCCGA AGCTGAGGCC CGGAGCAGCA CGCACGGGCG GGAGCGCAGC CAGGCAGAGC 300

CCTCCGAGCG GCGCGTGAAG CGGGAGAAGC GCGATGACGG CTACGAGGCC GCTGCCAGCT 360

CCAAAACTAG CTCAGGCGAT GCCTCCTCAC TCAGCATCGA GGAGACTAAC AAACTCCGGG 420

CAAAGTTGGG GCTGAAACCC TTGGAGGTTA ATGCCATCAA GAAGGAGGCG GGCACCAAGG 480

AGGAGCCCGT GACAGCTGAT GTCATCAACC CTATGGCCTT GCGACAGCGA GAGGAGCTGC 540

GGGAGAAGCT GGCGGCTGCC AAGGAGAAGC GCCTGCTGAA CCAAAAGCTG GGGAAGATAA 600

AGACCCTAGG AGAGGATGAC CCCTGGCTGG ACGACACTGC AGCCTGGATC GAGAGGAGCC 660

GGCAGCTGCA GAAGGAGAAG GACCTGGCAG AGAAGAGGGC CAAGTTACTG GAGGAGATGG 720

ACCAAGAGTT TGGTGTCAGC ACTCTGGTGG AGGAGGAGTT CGGGCAGAGG CGGCAGGACC 780

TGTACAGTGC CCGGGACCTG CAGGGCCTCA CCGTGGAGCA TGCCATTGAT TCCTTCCGAG 840

AAGGGGAGAC AATGATTCTT ACCCTCAAGG ACAAAGGCGT GCTGCAGGAG GAGGAGGACG 900

TGCTGGTGAA CGTGAACCTG GTGGATAAGG AGCGGGCAGA GAAAAATGTG GAGCTGCGGA 960

AGAAGAAGCC TGACTACCTG CCCTATGCCG AGGACGAGAG CGTGGACGAC CTGGCGCAGC 1020

AAAAACCTCG CTCTATCCTG TCCAAGTATG ACGAAGAGCT TGAAGGGGAG CGGCCACATT 1080

CCTTCCGCTT GGAGCAGGGC GGCACGGCTG ATGGCCTGCG GGAGCGGGAG CTGGAGGAGA 1140

TCCGGGCCAA GCTGCGGCTG CAGGCTCAGT CCCTGAGCAC AGTGGGGCCC CGGCTGGCCT 1200

CCGAATACCT CACGCCTGAG GAGATGGTGA CCTTTAAAAA GACCAAGCGG AGGGTGAAGA 1260

AAATCCGCAA GAAGGAGAAG GAGGTAGTAG TGCGGGCAGA TGACTTGCTG CCTCTCGGGG 1320

ACCAGACTCA GGATGGGGAC TTTGGTTCCA GACTGCGGGG ACGGGGTCGC CGCCGAGTGT 1380

CCGAAGTGGA GGAGGAGAAG GAGCCTGTGC CTCAGCCCCT GCCGTCGGAC GACACCCGAG 1440

TGGAGAACAT GGACATCAGT GATGAGGAGG AAGGTGGAGC TCCACCGCCG GGGTCCCCGC 1500

AGGTGCTGGA GGAGGACGAG GCGGAGCTGG AGCTGCAGAA GCAGCTGGAG AAGGGACGCC 1560

GGCTGCGACA GTTACAGCAG CTACAGCAGC TGCGAGACAG TGGCGAGAAG GTGGTGGAGA 1620

TTGTGAAGAA GCTGGAGTCT CGCCAGCGGG GCTGGGAGGA GGATGAGGAT CCCGAGCGGA 1680

AGGGGGCCAT CGTGTTCAAC GCCACGTCCG AGTTCTGCCG CACCTTGGGG GAGATCCCCA 1740

CCTACGGGCT GGCTGGCAAT CGCGAGGAGC AGGAGGAGCT CATGGACTTT GAACGGGATG 1800

AGGAGCGCTC AGCCAACGGT GGCTCCGAAT CTGACGGGGA GGAGAACATC GGCTGGAGCA 1860

CGGTGAACCT GGACGAGGAG AAGCAGCAGC AGGATTTCTC TGCTTCCTCC ACCACCATCC 1920

TGGACGAGGA ACCGATCGTG AATAGGGGGC TGGCAGCTGC CCTGCTCCTG TGTCAGAACA 1980

AAGGGCTGCT GGAGACCACA GTGCAGAAGG TGGCCCGGGT GAAGGCCCCC AACAAGTCGC 2040

TGCCCTCAGC CGTGTACTGC ATCGAGGATA AGATGGCCAT CGATGACAAG TACAGCCGGA 2100

GGGAGGAATA CCGAGGCTTC ACACAGGACT TCAAGGAGAA GGACGGCTAC AAACCCGACG 2160

TTAAGATCGA ATACGTGGAT GAGACGGGCC GGAAACTCAC ACCCAAGGAG GCTTTCCGGC 2220

AGCTGTCGCA CCGCTTCCAT GGCAAGGGCT CAGGCAAGAT GAAGACAGAG CGGCGGATGA 2280

AGAAGCTGGA CGAGGAGGCG CTCCTGAAGA AGATGAGCTC CAGCGACACG CCCCTGGGCA 2340

CCGTGGCCCT GCTCCAGGAG AAGCAGAAGG CTCAGAAGAC CCCCTACATC GTGCTCAGCG 2400

GCAGCGGCAA GAGCATGAAC GCGAACACCA TCACCAAGTG ACAGCGCCCT CCCGTAGTCG 2460

GCCCTGCCTC AACCTTCATA TTAAATAAAG CTCCCTCCTT ATTTTTAAAA AAAAAAAAAA 2520

AAAAAAA 2527

## CLAIMS

1. A DNA encoding a protein having the amino acid sequence shown in SEQ ID NO: 1 or a variant protein thereof in which one or more amino acid residues are substituted, deleted or added, said protein and variant protein thereof being capable of yielding, through its intracellular decomposition, peptide fragment(s) which can bind to major histocompatibility complex (MHC) class I antigen and which can be recognized by T cells in such binding state.

2. A DNA which comprises the base sequence shown in SEQ ID NO: 2, or a variant DNA which hybridizes to said DNA under stringent conditions, the protein produced by expression of said DNA and variant DNA being capable of yielding, through its intracellular decomposition, peptide fragment(s) which can bind to MHC class I antigen and which can be recognized by T cells in such binding state.

3. A medicine comprising DNA of claim 1 or 2 as an active ingredient.

4. An expression plasmid comprising DNA of claim 1 or 2.

5. A transformant transformed with the expression plasmid of claim 4.

6. A tumor antigen protein produced by expression of DNA of claim 1 or 2.

7. A tumor antigen peptide comprising part of the protein of claim 6, which can bind to MHC class I antigen to be recognized by T cells, or a derivative thereof having functionally equivalent properties.

8. A tumor antigen peptide of claim 7 which comprises all or

part of the amino acid sequence of positions 749-757, 736-744, 785-793, or 690-698 in the amino acid sequence of SEQ ID NO: 1, or a derivative thereof having functionally equivalent properties.

9. A medicine comprising, as an active ingredient, the tumor antigen protein of claim 6, the tumor antigen peptide or derivative thereof defined in claim 7 or 8.

10. An antibody which specifically binds to the tumor antigen protein of claim 6 or the tumor antigen peptide of claim 7 or 8.

11. A DNA comprising 8 or more bases having a sequence complementary to a coding or 5' non-coding sequence of DNA having the base sequence shown in SEQ ID NO: 2, an RNA corresponding to said DNA, or a chemically modified variant thereof.

## ABSTRACT

DNA encoding a protein having the amino acid sequence shown in SEQ ID NO: 1 or a variant protein thereof in which one or more amino acid residues are substituted, deleted or added, said protein or variant protein thereof being capable of yielding, through its intracellular decomposition, peptide fragment(s) which can bind to major histocompatibility complex (MHC) class I antigen and which can be recognized by T cells in such binding state, medicines comprising said DNA as an active ingredient, expression plasmids comprising said DNA, transformants transformed with said expression plasmids, as well as tumor antigen proteins and tumor antigen peptides produced by expression of said DNA.

# BIRCH, STEWART, KOLASCH & BIRCH, LLP

**PLEASE NOTE: YOU MUST COMPLETE THE FOLLOWING:**

## COMBINED DECLARATION AND POWER OF ATTORNEY FOR PATENT AND DESIGN APPLICATIONS

ATTORNEY DOCKET NO. 20-4491P

As a below named inventor, I hereby declare that: my residence, post office address and citizenship are as stated next to my name; that I verily believe that I am the original, first and sole inventor ( if only one inventor is named below) or an original, first and joint inventor (if plural inventors are named below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

**Insert Title:** TUMOR ANTIGEN PROTEINS, GENES THEREFOR, AND TUMOR ANTIGEN PEPTIDES

**Fill in Appropriate Information - For Use Without Specification Attached:**

the specification of which is attached hereto. If not attached hereto,

the specification was filed on _____ as United States Application Number _____; and /or

the specification was filed on June 4, 1997 as PCT International Application Number PCT/JP97/01893; and was amended under PCT Article 19 on _____ (if applicable)

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, §1.56.

I do not know and do not believe the same was ever known or used in the United States of America before my or our invention thereof, or patented or described in any printed publication in any country before my or our invention thereof or more than one year prior to this application, that the same was not in public use or on sale in the United States of America more than one year prior to this application, that the invention has not been patented or made the subject of an inventor's certificate issued before the date of this application in any country foreign to the United States of America on an application filed by me or my legal representatives or assigns more than twelve months (six months for designs) prior to this application, and that no application for patent or inventor's certificate on this invention has been filed in any country foreign to the United States of America prior to this application by me or my legal representatives or assigns, except as follows.

I hereby claim foreign priority benefits under Title 35, United States Code, §119 (a)-(d) of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

**Insert Priority Information: (if appropriate)**

| Prior Foreign Application(s) (Number) | (Country) | (Month/Day/Year Filed) | Priority Claimed |
|---|---|---|---|
| 168429/1996 | Japan | June/07/1996 | ☒ Yes ☐ No |
| 287572/1996 | Japan | Oct./08/1996 | ☒ Yes ☐ No |
| 330424/1996 | Japan | Nov./25/1996 | ☒ Yes ☐ No |
| | | | ☐ Yes ☐ No |
| | | | ☐ Yes ☐ No |

I hereby claim the benefit under Title 35, United States Code, §119(e) of any United States provisional application(s) listed below.

**Insert Provisional Application(s): (if any)**

(Application Number) _____ (Filing Date) _____

(Application Number) _____ (Filing Date) _____

All Foreign Applications, if any, for any Patent or Inventor's Certificate Filed More Than 12 Months (6 Months for Designs) Prior To The Filing Date of This Application:

**Insert Requested Information: (if appropriate)**

| Country | Application No. | Date of Filing (Month/Day/Year) |
|---|---|---|
| | | |
| | | |

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, §1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application:

**Insert Prior U.S. Application(s): (if any)**

| (Application Number) | (Filing Date) | (Status - patented, pending, abandoned) |
|---|---|---|
| | | |
| | | |

I hereby appoint the following attorneys to prosecute this application and/or an international application based on this application and to transact all business in the Patent and Trademark Office connected therewith and in connection with the resulting patent based on instructions received from the entity who first sent the application papers to the attorneys identified below, unless the inventor(s) or assignee provides said attorneys with a written notice to the contrary:

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

1-00

**Full Name of First or Sole Inventor:**
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**Insert Date This Document is Signed**
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**Insert Citizenship**
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**Full Name of Fifth Inventor, if any** see above

| GIVEN NAME | FAMILY NAME | INVENTOR'S SIGNATURE | DATE* |
|---|---|---|---|
| | | | |

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CITIZENSHIP:

POST OFFICE ADDRESS (Complete Street Address including City, State & Country):

* DATE OF SIGNATURE